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Wash water disinfection of a full-scale leafy vegetables washing process with hydrogen peroxide and the use of a commercial metal ion mixture to improve disinfection efficiency

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ABSTRACT

Hydrogen peroxide (H_2O_2) was used to maintain the microbial wash water quality of a full-scale leafy vegetables (radicchio, sugar loaf, curled endive, lollo, lollo rosso) wash water process. Despite addition of 300 L/h of 1.8% H_2O_2 to a 450 L washing bath (333 ± 50 kg/h fresh-cut produce introduction speed), the H_2O_2 quickly decreased and a lower wash water contamination of aerobic psychrotrophic plate count (APC) and enterococci than without addition of H_2O_2 could not be maintained. There was no significant difference between the APC on fresh-cut leafy vegetables washed with H_2O_2 and those washed with water.

In a second part, lab-scale experiments were performed to assess the impact of a commercial metal ion formulation (Bacsan®, containing a. o. Cu^{2+}, Zn^{2+}, Ag^{+}) on the stability of H_2O_2 in artificial wash water, made from iceberg lettuce and tap water. Bacsan improved the stability of H_2O_2 in artificial lettuce wash water and fresh-cut leafy vegetables wash water from a processing company and synergistically increased the disinfection efficiency of APC and *Escherichia coli* (*E. coli*) compared to H_2O_2 or Bacsan. Increasing chemical oxygen demand (COD) had detrimental effect on the H_2O_2 stability and disinfection efficiency. Addition of Ag^{+} to Bacsan further synergistically enhanced the H_2O_2 stability.

H_2O_2 is not suited as an *in situ* wash water disinfectant to avoid cross-contamination in fresh-cut leafy vegetables washing processes due to the slow water disinfection kinetics and the rapid H_2O_2 consumption. However, H_2O_2/Bacsan shows potential for use in off-line processes.

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1. Introduction

Among fresh produce, leafy vegetables are one of the commodities most frequently implicated with food disease outbreaks, the culprit most often being *Escherichia coli* O157: H7 or *Salmonella* spp. (Olaimat & Holley, 2012; Tomas-Callejas et al., 2012). Washing of fresh-cut lettuce is often the only processing step able to reduce the microbial load (Artés, Gómez, Aguayo, Escalona, & Artés-Hernández, 2009). Current washing treatments with the purpose of decontaminating fresh-cut produce for microbial safety or quality reasons, have evolved from processes that were originally developed to remove soil from whole produce, to a water disinfection process for removal of microbial targets from fresh-cut produce (Sapers, 2001). The success of these washing processes to remove naturally present microorganisms from fresh-cut produce is limited (1–3 log reduction), i.e. microbial reductions occur but total removal cannot be achieved. The access of sanitizers to the target microorganisms is hindered by the presence of microorganisms in biofilms, attachment near and within stomata, and internalization through cut surfaces and other tissue wounds. Therefore it is preferable to avoid contamination wherever possible by implementing good agricultural and manufacturing practices during the production and processing of fresh produce (Holvoet, Jacxsens, Sampers, & Uyttendaele, 2012; Holvoet, Sampers, Callens, Dewulf, & Uyttendaele, 2013; Keskinen, Burke, & Annous, 2009; López-Gálvez, Gil, Truchado, Selma, & Allende, 2010; Sapers, 2001). The post-harvest washing water is a vehicle for microbial cross-contamination and to counter this an *in situ* wash

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water disinfection can be performed. Water disinfection can also be used to treat the wash water before reusing it (i.e. reconditioning) for a similar or different purpose. The efficiency of wash water disinfection is not limited by the issues that plague decontamination, but the effectiveness of chemical oxidants (a. o. chlorine, chlorine dioxide, ozone, H_2O_2, peracetic acid) is hindered by the presence of organic matter in the wash water, the degree depending on the properties of the chemical oxidant (Van Haute, Sampers, Jacxsens, & Uyttendaele, 2013).

H_2O_2 does not produce toxic fumes in the worker space and is an environmentally friendly alternative to chlorine for decontamination of fresh produce, as it breaks down in water and oxygen (Tofant, Vucemilo, Pavicic, & Milic, 2006), and does not form carcinogenic disinfection byproducts (USEPA, 1997; Van Haute, Sampers, Jacxsens, et al., 2013). Considerable research has been conducted on the use of H_2O_2 as produce decontamination agent against bacterial and viral indicator organisms, pathogenic bacteria, or spoilage microflora on fresh (-cut) fruit and vegetables (Parish et al., 2003; Ukuku, Bari, & Kawamoto, 2012), among which some experiments have been performed on leafy vegetables (Allwood, Malik, Hedberg, & Goyal, 2004; Hadjok, Mittal, & Warriner, 2008; Huang & Chen, 2011; Li et al., 2011; Lin, Moon, Doyle, & McWatters, 2002). On the contrary, its use as a water disinfectant to control the wash water quality of fresh produce washing processes is virtually unexplored. Earlier water disinfection studies that focused on inactivating vegetative bacteria, bacterial spores, viruses, or protozoa have shown that H_2O_2 by itself is a slow acting water disinfectant, requiring high dosages and contact times for microbial inactivation (Barbee, Weber, Sobsey, & Rutala, 1999; Raffellini, Guerrero, & Alzamora, 2008; Raffellini, Schenk, Guerrero, & Alzamora, 2011; Toledo, Escher, & Ayres, 1973; Weir, Pokorny, Carreno, Trevors, & Lee, 2002). Combined with Ag^{+} and $Cu^{1\text{ or }2+}$, performance of H_2O_2 can be enhanced (Batterman, Zhang, & Wang, 2000; Orta De Velasquez, Yanez-Noguez, Jimenez-Cisneros, & Luna Pabello, 2008; Pedahzur et al., 2000; Pedahzur, Shuval, & Ulitzur, 1997).

In this study, the use of H_2O_2 to maintain the microbial wash water quality in a full-scale industrial fresh-cut leafy-vegetables washing process was assessed. To the knowledge of the authors, this is the first published study that utilizes H_2O_2 as wash water sanitizer in a full-scale washing process of fresh-cut leafy vegetables. Also, lab-scale experiments were performed to assess the use of Bacsan (containing a. o. Cu^{2+}, Ag^{+}, and Zn^{2+}) to improve the H_2O_2 disinfection efficiency in post-harvest water disinfection processes.

2. Materials and methods

2.1. Water disinfection in a fresh-cut leafy vegetables processing company

2.1.1. Experimental setup

Experiments were executed in a Belgian fresh-cut leafy vegetables processing company. First, a run was executed without addition of water disinfectant, i.e. the 'blank' run. A batch of 400 kg mixed salad was processed, containing radicchio (33%), sugar loaf (*Chicorium intybus*) (33%) and curled endive (33%). The leafy vegetables were cut (in pieces of 1 by 5 cm), and transported through two subsequent immersion washing baths (washing bath 1: WB1 and washing bath 2: WB2) with a volume of 450 L each, and a leafy vegetable residence time of 1 min in each washing bath. The washing system consisted of bubble washers, i.e. production of agitation in the washing baths by air bubble injection through underwater air nozzles. Subsequently they were transported by a conveyer belt to a centrifuge for dewatering, followed by a weighing unit (computer controlled weight proportioning scales). Both washing baths were filled with bore hole water, cooled on beforehand to 2 °C. During the washing process, 300 L/h of bore hole water was added to each of the washing baths. Wash water was recirculated within washing baths but not between washing baths. The only water that was transferred from WB1 to WB2 was the water that was attached to the transferred lettuce. Two wash water disinfection experiments were performed. In both experiments, the same types of leafy vegetables were processed during the wash water disinfection experiments of which the first batch (467 ± 55 kg) was the same leafy vegetables mix as in the blank runs. In addition, a second batch (258 ± 31 kg) was processed, consisting of white lollo (*Lactuca sativa* cv. Lollo Bianco) (50%) and lollo rosso (*L. sativa* cv. Lollo Rosso) (50%). For each type of leafy vegetable and experiment, the crops originated from the same farm, and the crops were processed at the day of harvest. On average leafy vegetables were washed at 333 ± 50 kg/h. In the disinfection experiments, WB1 was operated identically to the blank runs. In the first disinfection experiment, WB2 was filled with 1.8% H_2O_2 (i.e. 4% EcoClearProx, ABT Belgium, Belgium) and 300 L/h 1.8% H_2O_2 of bore hole water was added. In the second disinfection experiment, WB2 was filled with 1.8% H_2O_2 and 300 L/h of bore hole water was added. During processing, 300 L/h of wash water was tapped from the washing bath and 5.4 L/h H_2O_2 was dosed (again to obtain addition of 1.8% H_2O_2/L) and sent through a low pressure UV-C system (Aquada 2, Wedeco, Belgium; 55 W) with fluence of 240 mJ/cm^2 at a flow of 300 L/h and 98% UV 254 nm transmittance/cm, before recirculation to WB2.

2.1.2. Sampling in the fresh-cut leafy vegetable processing company

Samples of the fresh-cut leafy vegetables, water samples from WB1 and WB2, and samples from the food contact surfaces of the conveyer belt and the weighing unit were taken five times throughout the processing: at the start of batch 1, at the middle of batch 1, at the end of batch 1 = start of batch 2, in the middle of batch 2, at the end of batch 2. About 250 g of fresh-cut leafy vegetables was sampled and put directly into a sterile stomacher bag. For sampling the raw material, each lettuce type was sampled separately per batch, and averaged as the microbial count of the raw material. The water samples were collected into a sterile 1 L bottle according to ISO 19458:2006 (ISO, 2006). Excess H_2O_2 was quenched with sterile $Na_2S_2O_3$. The food contact surfaces were sampled with sterile swabs. Aseptic templates covering 50 cm^2 were used and a sterile swab moistened in 5 mL of buffered peptone water was used to swab a delimited area vertically, horizontally, and diagonally. All the samples were stored and transported in the dark at <4 °C to the lab for further handling and subsequent microbial analysis within 12 h. For each measuring point two independent samples were taken. At each time point and operation unit, water and food contact surfaces were sampled at two consistent points, and each of the two samples for raw materials screening originated from two crops.

2.1.3. Microbial analyses

For the fresh-cut leafy vegetables samples and food contact surfaces, APC and *E. coli* were enumerated, whereas in the water also enterococci were enumerated. For the fresh-cut leafy vegetables samples, 10 g of fresh-cut leafy vegetables was weighed in a stomacher bag and homogenized for 1 min in 90 mL buffered peptone water. The enumeration of APC was done with the reference method ISO 4833:2003 (ISO, 2003), with the exception that the plates were incubated at 22 °C for five days instead of at 30 °C for 3 days. *E coli* was enumerated with the pour plate method on RAPID'*E.coli 2* agar (BioRad, France), a selective chromogenic medium, incubated for 24 h at 37 °C. For the water and food contact

surface samples, APC was measured according to ISO 6222:1999 and incubated for 3 days at 22 °C (ISO, 1999). The enumeration of *E. coli* was done according to ISO 9308-1 (i.e. membrane filtration) with the exception that the tergitol 7 medium was replaced by RAPID'*E.coli* 2 agar (Biorad, France) (ISO, 2000a). The detection and enumeration of enterococci was performed using the membrane filtration method ISO 7899-2 (ISO, 2000b).

2.1.4. Physicochemical parameters

Alkalinity was determined with acid titration, turbidity with a turbidimeter (HI98703, HANNA Instruments, Belgium), COD according to the small-scale sealed-tube method (LCI 400, Hach Lange, Belgium). H_2O_2 concentration, pH and T were determined at the fresh-cut leafy vegetables processing company. H_2O_2 was determined with the spectrophotometric I_3^- method by Klassen, Marchington, and McGowan (1994). H_2O_2 was determined immediately after sampling, to avoid further consumption due to reaction with the water matrix components.

2.2. Water disinfection in standardized wash water

2.2.1. Standardized wash water

The outer leaves of the iceberg lettuce (*L. sativa* L.) were removed. The leaves were cut into pieces of about 3 cm and 67 g of the cut salad was put in a stomacher bag to which 200 mL of tap water was added. The mixture was homogenized for 2 min. The COD of this suspension was determined, and subsequently, this mixture was diluted with tap water, to obtain standardized wash water (SWW) with the desired COD.

2.2.2. Industrial wash water

Industrial wash water was collected at a Norwegian fresh-cut leafy vegetables processing company. The water was collected immediately after a batch of mixed lettuce, i.e. iceberg lettuce, rucola (*Eruca sativa*) and radicchio, had been washed with tap water.

2.2.3. Bacterial inoculation

E. coli ATCC 25922 was grown in nutrient broth (Oxoid, France) for 24 h at 37 °C. The *E. coli* cells were washed in phosphate-buffered saline and subsequently added to the SWW to obtain 5–6 log CFU/mL.

2.2.4. Physicochemical parameters

COD and H_2O_2 were measured as described above. The Cu^{2+} ion concentration in SWW was measured with a test kit (MD 200 2IN1 copper, Lovibond, Germany), based on the reduction of Cu^{2+} to Cu^+, the reaction of Cu^+ with bicinchoninic acid, followed by spectrophotometric measurement of the formed complex. Free chlorine was measured as described by Van Haute, Sampers, Holvoet, & Uyttendaele (2013).

2.2.5. Disinfection experiments

H_2O_2 was diluted from a 30% stock-solution (Fluka Analytical, Germany). Bacsan (Labola, Norway) is a patented, commercial formulation from Aqua Chemical Nutrients, marketed as water disinfectant and containing a. o. Cu, Ag, and Zn. The content of the Bacsan solution was analyzed with inductive coupled plasma emission spectrometry to determine the actual metal ion concentrations and were found to be: 84.2 ± 1.1 g/L Cu, 7.3 ± 0.2 mg/L Ag, 23.7 ± 0.4 g/L Zn, 24.0 ± 0.4 mg/L Al, and 56.0 ± 0.2 g/L NO_3^-. 100 mL of continuously mixed, inoculated SWW at 4 ± 2 °C was exposed to 500 mg/L of H_2O_2, with or without the addition of 2 or 10 mg/L Bacsan-Cu (expressed as mg/L Cu^{2+} in Bacsan), or to 500 mg/L of H_2O_2 with the addition of 10 mg/L Cu^{2+} from $CuSO_4.5H_2O$ (Merck, Germany), or to 10 mg/L Bacsan-Cu without H_2O_2. H_2O_2 residual concentration was measured after 5, 30, and 120 min. Microbial samples were taken after 30 and 120 min and immediately quenched with $Na_2S_2O_3$. For each treatment and chosen COD level of SWW, 3 independent experiments were executed. The industrial fresh-cut leafy vegetables wash water was similarly treated as the SWW, except for the *E. coli* inoculation which was not executed. Also, exposure of SWW to 500 mg/L of H_2O_2 with addition of 2 mg/L Bacsan-Cu and 0.1 mg/L Ag^+ from $AgNO_3$ (Sigma–Aldrich, Germany) or to 500 mg/L of H_2O_2 with addition of 10 mg/L Bacsan-Cu and 1 mg/L Ag^+ was assessed for H_2O_2 stability in SWW.

2.2.6. Microbial analyses

APC was enumerated with the pour plate method on Water plate count agar (Oxoid, England) (incubated for 3 days at 22 °C) and *E. coli* with the pour plate method, using RAPID'*E. coli* 2 agar (Biorad, France) (incubated for 24 h at 37 °C).

2.2.7. Assessment of the interaction of catalase and Bacsan

For investigating the effect of Bacsan and pH on the H_2O_2 consumption caused by the enzyme catalase, SWW was rapidly heated to 80 °C and maintained at 80 °C for 10 min to inactivate catalase (Anderson, 2002; Hirvi, Griffiths, McKellar, & Modler, 1996). Thereafter, the SWW was rapidly cooled to 4 °C. The heated and unheated SWW were treated with 630 mg/L H_2O_2 (with or without 10 mg/L Bacsan-Cu) and also with 100 mg/L free chlorine for comparison with a disinfectant that is no specific target of an enzyme. Free chlorine was diluted from a chlorine stock solution (28.4 g/L NaOCl, La Croix, Belgium).

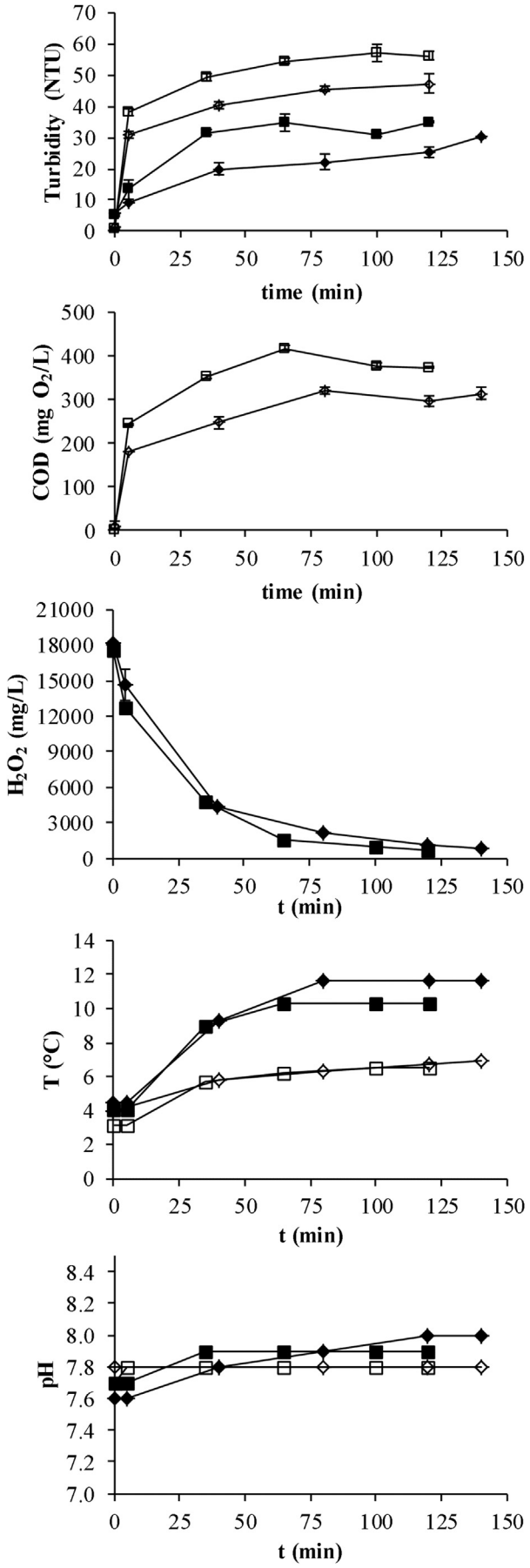
Also, experiments using pure catalase from bovine liver (Sigma–Aldrich, Norway) were performed. H_2O_2 was diluted in 0.05 mol/L phosphate buffer with pH 5.5, 6.0 or 7.2 to a final concentration of 590 mg/L H_2O_2. Preliminary tests were done at pH 5.5 and pH 7.2 by adding 0, 2, 10 mg/L Bacsan-Cu, or 10 mg/L Cu^{2+} (as $CuSO_4.5H_2O$) to the buffered H_2O_2 solutions, to which catalase was added to a final concentration of 1.5 mg/L. The consumption of H_2O_2 caused by the added catalase was assessed by measuring the H_2O_2 residual concentration after 5 and 30 min, at 4 ± 2 °C, and under continuous mixing. More detailed experimentation was done with 0 and 10 mg/L Bacsan-Cu in the presence of 590 mg/L H_2O_2 in buffered solutions at pH 5.5, 6.0, and 7.2. The H_2O_2 residual concentration was measured after 5 min and 30 min. The experiments were performed at 4 ± 2 °C and repeated 3 times.

2.2.8. Oxidative browning

Lettuce washing experiments were executed to determine the effect of the treatments (i.e. 500 mg/L H_2O_2 with or without Bacsan) on oxidative browning of the lettuce and consisted of washing 30 g of cut lettuce with mechanical agitation for 2 min at 4 ± 2 °C, followed by centrifugation and storage in sterile plastic boxes at 4 ± 2 °C. After 3, 4, and 5 days the lettuce samples were observed for visible traces of enzymatic browning.

2.3. Statistics

Statistical analysis was performed with SPSS statistics 21 and Microsoft Excel. Comparison of parameter levels was done with one-way ANOVA or Brown-Forsythe when equal variance could not be assumed. Group comparison was done with post-hoc tests (Tukey or Games-Howell). For comparing means of parameters, the Mann Whitney-U and Wilcoxon-signed rank tests were used for unpaired and paired samples respectively. A level of significance of $p \leq 0.05$ was chosen for all statistical analyses.



3. Results

3.1. H_2O_2 wash water disinfection in a fresh-cut leafy vegetables processing company

Initially, the washing process turbidity and COD increased rapidly and subsequently, in general, the increase diminished as a function of time (Fig. 1). The turbidity in WB1 was significantly higher than in WB2 during the trials. The COD in WB2 was not measured due to the interference of H_2O_2. The H_2O_2 concentration rapidly decreased during the washing process (Fig. 1), and a significant negative correlation between turbidity and H_2O_2 concentration ($r^2 = 0.608$; $p < 0.0005$) was observed. The temperature increased to higher values in WB2 compared to WB1. The pH of the bore hole water was 7.4 ± 0.1. The pH value rose to slightly higher values in WB2 compared to WB1 towards the end of the water disinfection experiments, though not significantly (Fig. 1). The alkalinity did not change significantly as a function of time and was not significantly different between treatments nor washing baths, with 6.36 ± 0.10, 6.38 ± 0.14 and 6.51 ± 0.16 mmol/L bicarbonate in the bore hole water, WB1 and WB2 respectively, indicating it originated predominately from the bore hole water itself.

The *E. coli* contamination was below the limit of detection at all times and locations, i.e. <1 log CFU/g on the fresh-cut leafy vegetables, <0.3 log CFU/100 mL in the water and <0.7 log CFU/50 cm^2 on the conveyer belt and the weighing unit. APC and enterococci contamination was significantly higher in WB1 compared to WB2 in all experiments (Fig. 2). To assess the impact of the water disinfection treatments, the differences between the measured contamination in both washing baths was calculated (WB1 − WB2) in order to be able to compare the wash water disinfection efficiency of the treatments and to incorporate fluctuations in transfer of microorganisms from fresh-cut leafy vegetables to the washing baths between the different water disinfection trials. These differences were significantly higher for both disinfection treatments compared to the blank during the first batch (first 60–80 min dependent on the batch size), i.e. the wash water contamination was significantly reduced with the 1.8% H_2O_2 (with or without UV) treatment. For enterococci, no significant reductions in the wash water were found. The gradually increasing APC concentration in the H_2O_2 treated wash water (Fig. 2b) reflects the declining H_2O_2 residual during the washing process (Fig. 1), despite the continuous addition of 300 L/h of 1.8% H_2O_2 in a 450 L washing bath, due to the build-up of organic matter in the washing bath, indicated in WB2 as increasing turbidity (Fig. 1). The APC and enterococci contamination after the UV/H_2O_2 unit was reduced to below the detection limit (<2 log CFU/100 mL and <0.3 log CFU/100 mL respectively) at all times. However, a decrease of the wash water contamination in WB2 compared to the 1.8% H_2O_2 treatment was not observed.

For batch 1, the initial APC load of the fresh-cut leafy vegetables was 7.1 ± 0.4, 6.8 ± 0.3, and 6.8 ± 0.2 log CFU/g for the blank run, 1.8% H_2O_2, and 1.8% H_2O_2 + UV respectively, whereas for batch 2, it was 7.4 ± 0.2 and 7.6 ± 0.2 log CFU/g for 1.8% H_2O_2 and 1.8% H_2O_2 + UV respectively. The APC load on the fresh-cut leafy vegetables was reduced significantly with 1.8% H_2O_2 (Fig. 3), with or without UV, but also with a water wash. The introduction of organic matter lowered the H_2O_2 concentration as the washing process advanced in time. However, processing time had no influence on decontamination efficiency of any of the treatments, and 1.8% H_2O_2 (with or without UV) did not improve the decontamination

Fig. 1. Turbidity, COD, H_2O_2, T, and pH during the washing bath trials with 1.8% H_2O_2, measured in WB 1(◊) and WB 2 (◆), 1.8% H_2O_2 + UV, measured in WB 1 (□) and WB 2 (■) ($n = 3$).

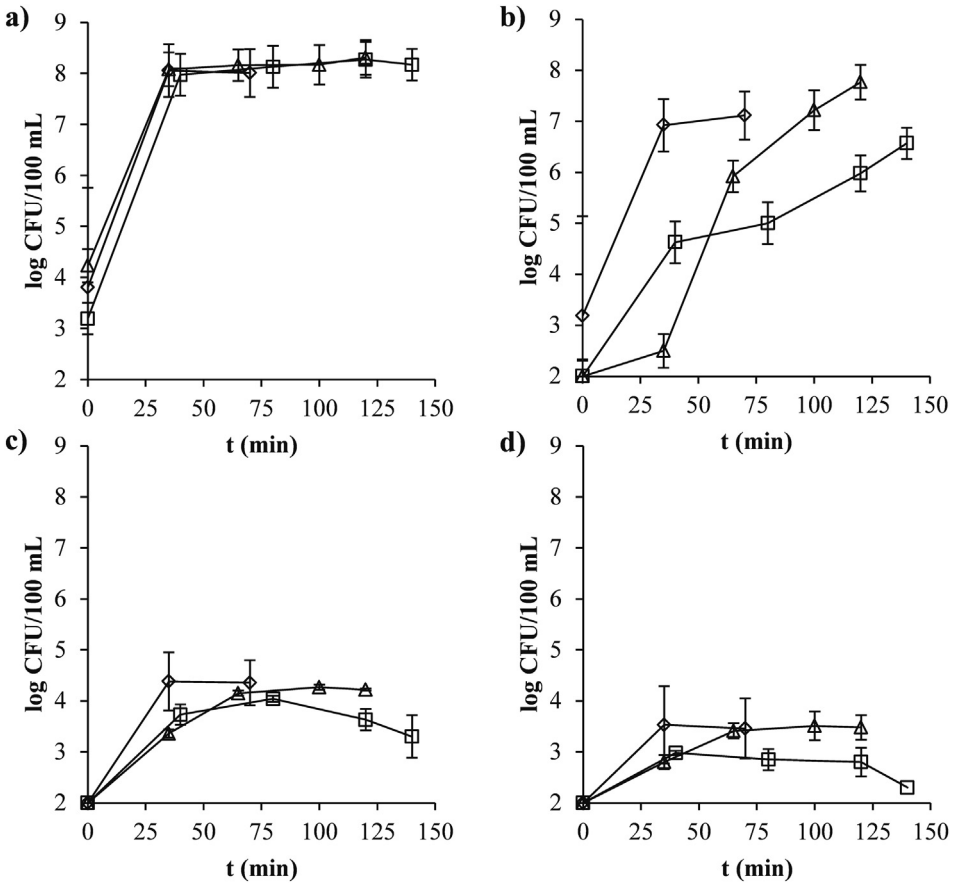


Fig. 2. Washing bath contamination of APC during the washing trials in the screened company in a) WB1 and b) WB2 and enterococci in c) WB1 and d) WB2; during the blank run (◊), when WB2 was treated with 1.8% H_2O_2 (□), when WB2 was treated with 1.8% H_2O_2 + UV (△) ($n = 2$).

efficiency (considering batch 1) compared to a water wash (Fig. 3). The APC contamination on the conveyer belt increased during processing from 3.1 to 4.8, 2.7 to 4.2 and 2.3 to 4.7 log CFU/50 cm^2 in the blank run, with 1.8% H_2O_2, and with 1.8% H_2O_2 + UV respectively, and on the weighing unit from 3.2 to 5, 2.3 to 4.7, and 3.5 to 5.5 log CFU/50 cm^2 respectively.

3.2. H_2O_2 stability in SWW

For all treatments, both COD and contact time had a significant detrimental influence on the H_2O_2 concentration in the SWW (Table 1). The rate of H_2O_2 consumption was lowest with H_2O_2 + 10 mg/L Bacsan-Cu. The consumption rate was lower with H_2O_2 + 2 mg/L Bacsan-Cu than with H_2O_2 at COD 497 and 848 mg O_2/L, whereas at COD 1830 mg O_2/L no difference was observed (Table 1). The stability of H_2O_2 in SWW of COD 789 mg O_2/L was higher with H_2O_2 + 10 mg/L Bacsan-Cu compared to H_2O_2 + 10 mg/L Cu^{2+} (from $CuSO_4$), which in turn was significantly higher after 30 min than in the absence of metal ions (Fig. 4). The addition of 0.1 mg/L Ag^+ to 2 mg/L Bacsan-Cu and 1 mg/L Ag^+ to 10 mg/L Bacsan-Cu in SWW of COD 753 mg O_2/L further enhanced the stability of initially added 500 mg/L H_2O_2 in a synergistic fashion (Fig. 5).

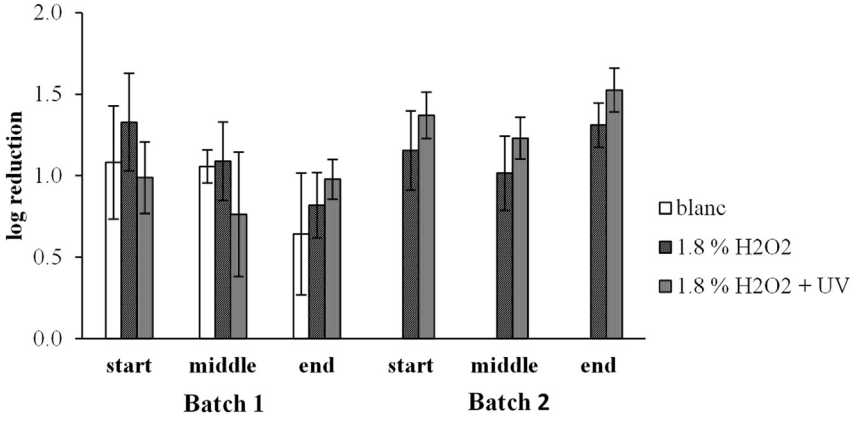


Fig. 3. APC reduction on the fresh-cut leafy vegetables due to the industrial washing processes in the screened company ($n = 2$).

Table 1
Microbial reduction and H_2O_2 concentration during water disinfection trials in SWW of varying COD and industrial wash water with 500 mg/L H_2O_2 and/or Bacsan dosage.

COD (mg O_2/L)	SWW									Industrial wash water		
	497 ± 7			848 ± 6 mg			1830 ± 21			509 ± 3		
t (min)	5	30	120	5	30	120	5	30	120	5	30	120
APC (log reduction)												
H_2O_2		0.7 ± 0.1	2.2 ± 0.5		0.5 ± 0.2	1.3 ± 0.3		0.0 ± 0.1	0.0 ± 0.2		0.5 ± 0.2	1.0 ± 0.1
H_2O_2 + 2 mg/L Bacsan-Cu		2.0 ± 0.4	2.7 ± 0.1		1.2 ± 0.2	1.3 ± 0.3		0.0 ± 0.2	−0.1 ± 0.3		0.4 ± 0.1	0.8 ± 0.1
H_2O_2 + 10 mg/L Bacsan-Cu		4.8 ± 0.1	5.0 ± 0.2		4.5 ± 0.2	4.5 ± 0.2		0.1 ± 0.1	0.9 ± 0.1		2.8 ± 0.2	3.3 ± 0.1
10 mg/L Bacsan-Cu		2.6 0.1	>2.7		0.8 ± 0.3	1.1 ± 0.2		0.1 ± 0.2	0.1 ± 0.1		0.3 ± 0.1	0.8 ± 0.1
***E. coli* (log reduction)**												
H_2O_2		0.7 ± 1.1	3.0 ± 0.1		0.6 ± 0.2	0.7 ± 0.2		0.0 ± 0.1	0.1 ± 0.1			
H_2O_2 + 2 mg/L Bacsan-Cu		3.8 ± 0.2	4.3 ± 0.5		3.0 ± 0.3	3.0 ± 0.3		0.2 ± 0.1	0.4 ± 0.1			
H_2O_2 + 10 mg/L Bacsan-Cu		>5	>5		>5	>5		1.2 ± 0.1	1.8 ± 0.2			
10 mg/L Bacsan-Cu		>2.5	>2.5		1.2 ± 0.2	1.9 ± 0.1		0.2 ± 0.1	0.1 ± 0.2			
H_2O_2 (mg/L)												
H_2O_2	314 ± 2	38 ± 1	1 ± 0.1	221 ± 2	1 ± 0.1	1 ± 0.1	94 ± 1	3 ± 1	1 ± 0.1	338 ± 3	40 ± 1	1 ± 0.1
H_2O_2 + 2 mg/L Bacsan-Cu	358 ± 1	57 ± 3	16 ± 2	241 ± 2	27 ± 2	1 ± 0.1	92 ± 2	6 ± 1	1 ± 0.1	352 ± 6	88 ± 1	35 ± 1
H_2O_2 + 10 mg/L Bacsan-Cu	374 ± 2	166 ± 9	88 ± 2	319 ± 3	143 ± 8	72 ± 2	157 ± 5	16 ± 1	2 ± 0.1	387 ± 7	202 ± 8	122 ± 1

The Cu^{2+} concentration in solution decreased only moderately after 120 min treatment time, i.e. 9.5 ± 0.7%, 3.6 ± 1.3%, and 5.6 ± 3.1% for H_2O_2 + 10 mg/L Bacsan-Cu, H_2O_2 + 2 mg/L Bacsan-Cu and 10 mg/L Bacsan-Cu respectively. The pH of the SWW was 7.3 ± 0.2. Addition of 500 mg/L H_2O_2 did not change the pH significantly, whereas addition of 10 mg/L Bacsan-Cu, H_2O_2 + 2 mg/L Bacsan-Cu, and H_2O_2 + 10 mg/L Bacsan-Cu decreased the pH with 0.3–0.7, 0.3 to 0.9, and 0.5 to 1.0 respectively, the pH drop increasing with decreasing COD of the SWW, most likely due to an increasing amount of pH buffering molecular species in SWW of higher COD. This pH drop was in part due to the low pH of the Bacsan stock-solution i.e. below the detection limit of the pH meter (pH 0).

When heating SWW of COD 819 mg O_2/L, the COD did not change significantly. Addition of 630 mg/L H_2O_2 to heated SWW led to an initial rapid decrease in the first 5 min, after which no considerable further consumption occurred during the remaining 25 min (Fig. 6a). Addition of Bacsan had no influence on the H_2O_2 consumption. In the unheated water however, Bacsan decreased the H_2O_2 consumption (as observed before). The H_2O_2 residual in heated SWW was considerably larger than in the unheated SWW, whereas for free chlorine the difference in consumption was much smaller (Fig. 6b).

To explain the increased stability of H_2O_2 in the presence of Cu^{2+} and to larger extent in the presence of Bacsan, the impact on the activity of bovine liver catalase activity was assessed. In the absence of catalase the H_2O_2 concentration remained constant in all the phosphate buffered solutions during the experimental period. The pH was measured and found to not be affected by the addition of H_2O_2 and Bacsan. Preliminary experiments (without repeats) showed the following order of H_2O_2 stability: H_2O_2 < H_2O_2 + 2 mg/L Bacsan-Cu ~ H_2O_2 + 10 mg Cu^{2+} (from $CuSO_4$) < H_2O_2 + 10 mg/L Bacsan-Cu (data not shown). More detailed experiments showed that at each pH value (5.5, 6, or 7.2), the consumption of H_2O_2 was significantly lower in the presence of 10 mg/L Bacsan-Cu than in absence of Bacsan (Fig. 7). The pH affected the consumption of H_2O_2 by catalase. The highest residual H_2O_2 concentration after 5 and 30 min was measured at pH 5.5 in the presence of 10 mg/L Bacsan-Cu (Fig. 7).

3.3. Water disinfection in SWW

At the start of the experiments in SWW, the APC was 6.0 ± 0.2 log CFU/mL averaged among all experiments, and the inoculated *E. coli* contamination was 5.4 ± 0.4 log CFU/mL. *E. coli* was more susceptible than APC to H_2O_2 combined with 2 mg/L or 10 mg/L Bacsan-Cu (Table 1). The inactivation of APC was significantly higher with H_2O_2 + 10 mg/L Bacsan-Cu compared to the other treatments, whereas for *E. coli*, the reduction was higher with H_2O_2 combined with 2 or 10 mg/L Bacsan-Cu compared to the other treatments. Exposure to increasing concentrations of COD had a detrimental influence on the inactivation of *E. coli* and APC. A significantly higher inactivation of APC and *E. coli* after 120 min

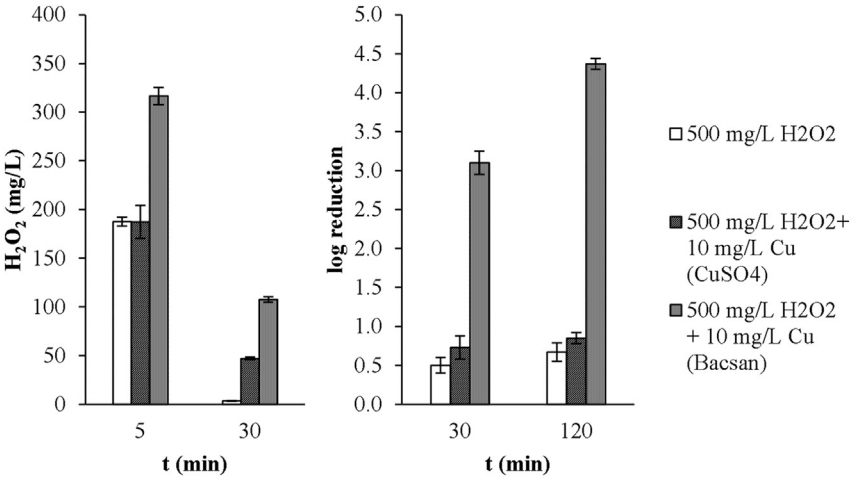


Fig. 4. Residual H_2O_2 and APC reduction in SWW of COD 789 ± 7 mg O_2/L when comparing treatment with 500 mg/L H_2O_2, H_2O_2 + 10 mg/L Cu^{2+} (as $CuSO_4$) and H_2O_2 + 10 mg/L Bacsan-Cu (n = 3).

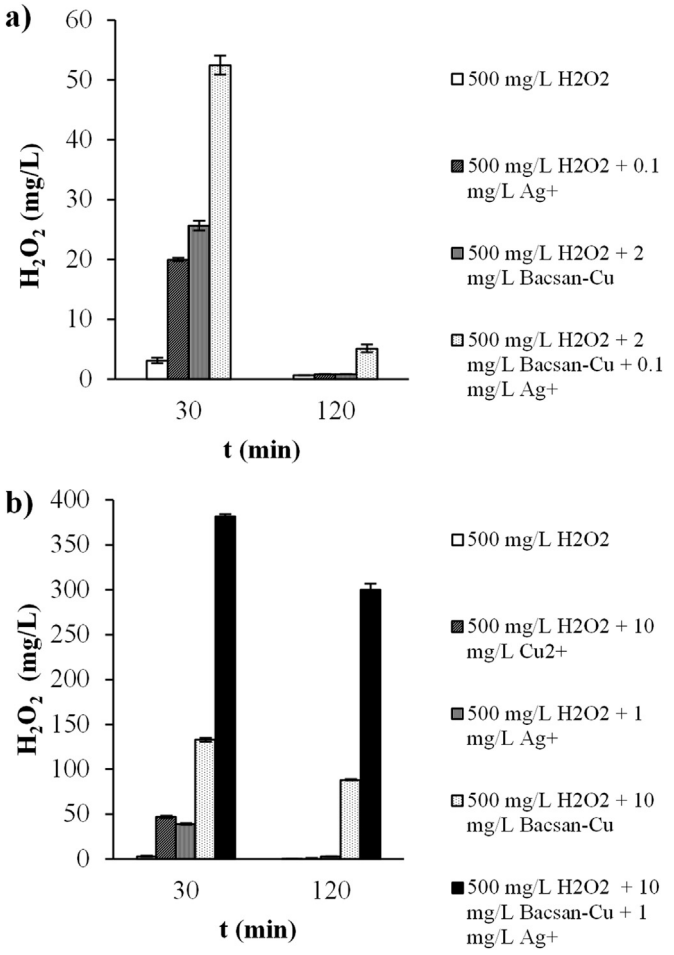


Fig. 5. H_2O_2 residual in function of time, when adding Ag^+ to Bacsan in SWW of COD 753 ± 5 mg O_2/L, a) combinations with 2 mg/L Bacsan-Cu and 0.1 mg/L Ag^+, b) combinations with 10 mg/L Bacsan-Cu and 1 mg/L Ag^+, ($n = 3$).

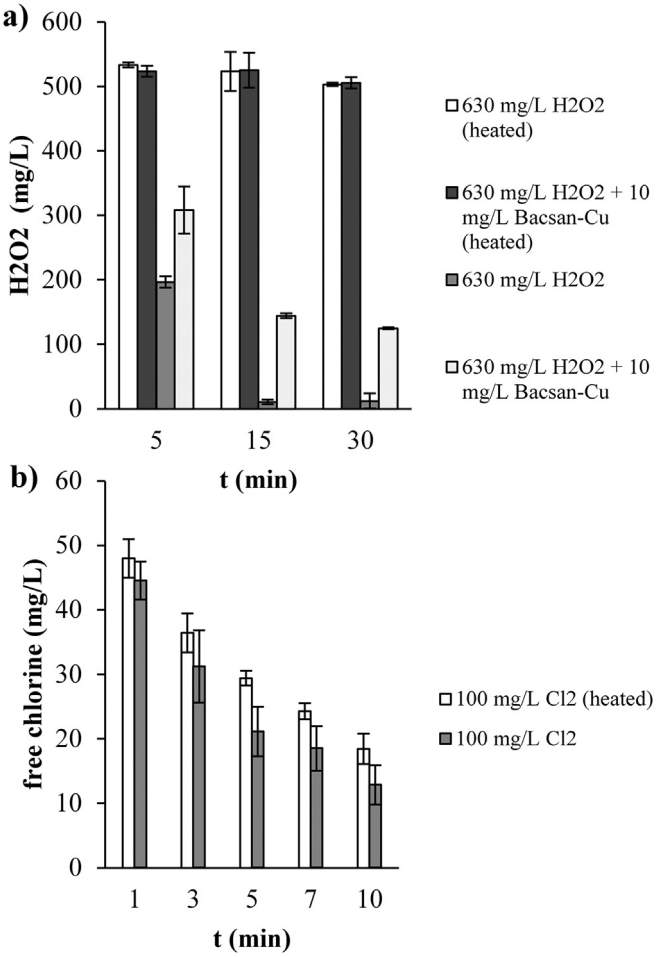


Fig. 6. a) Residual H_2O_2 concentration of initially added 630 mg/L H_2O_2 in SWW with COD 819 ± 10 mg O_2/L, with or without heating at 80 °C for 10 min, and b) residual free chlorine concentration of initially added 100 mg/L free chlorine in SWW with COD 634 ± 2 mg O_2/L, with or without heating at 80 °C for 10 min ($n = 3$).

compared to 30 min contact time was only observed at COD 1830 mg O_2/L with H_2O_2 + 10 mg/L Bacsan-Cu and at COD 497 mg O_2/L with H_2O_2, and of *E. coli* at COD 848 mg O_2/L with 10 mg/L Bacsan-Cu. The low improvement on disinfection efficiency in the interval 30–120 min contact time is attributed to the low remaining H_2O_2 residual (Table 1).

Synergy of H_2O_2 + 10 mg/L Bacsan-Cu was observed in SWW of COD 848 and 1830 mg O_2/L (except for APC after 30 min in COD 1830 mg O_2/L) (Table 1). In SWW of COD 497 mg O_2/L, observation of possible synergy was hindered by detection limit issues combined with overall higher inactivation due to the lower physico-chemical load of the SWW. Only the presence of synergy in the case of APC after 30 min could easily be observed. The reduction of APC in SWW of COD 789 mg O_2/L was higher with H_2O_2 + 10 mg/L Bacsan-Cu compared to H_2O_2 + 10 mg/L Cu^{2+} (as $CuSO_4$) (Fig. 4). Despite the H_2O_2 concentration being significantly higher after 30 min when adding Cu^{2+} than in the absence of Cu^{2+} (as $CuSO_4$), the APC inactivation with H_2O_2 + 10 mg/L Cu^{2+} was not significantly different from that obtained with only H_2O_2 (Fig. 4).

3.4. H_2O_2 consumption and water disinfection in industrial wash water from a processing company

3 CFU/100 mL *E. coli* were found in the industrial wash water and none were detected after the disinfection treatments. The inactivation of APC was lower in industrial wash water of COD 509 mg O_2/L compared to in SWW of COD 497 mg O_2/L (Table 1). Nonetheless, the H_2O_2 residual was significantly higher in the industrial wash water than in SWW after 30 and 120 min contact time with H_2O_2 + 2 and 10 mg/L Bacsan-Cu, but not with solely H_2O_2 compared to SWW. As in SWW, both the highest H_2O_2 stability and a synergistic APC inactivation were observed with 500 mg/L H_2O_2 + 10 mg/L Bacsan-Cu.

3.5. Impact of washing treatments on browning of stored fresh-cut iceberg lettuce

After washing the lettuce in the water disinfection solutions, some browning appeared after 3 days of storage in the fresh-cut lettuce, and considerable more browning was observed when washing in 10 mg/L Bacsan-Cu and H_2O_2 + 10 mg/L Bacsan-Cu compared with washing in water, 2 mg/L Bacsan-Cu, H_2O_2, and H_2O_2 + 2 mg/L Bacsan-Cu. When the lettuce was rinsed after treatment with 10 mg/L Bacsan-Cu (with or without H_2O_2), the amount of browning was similar to that of tap water treatment (and the other treatments) for the 5 days storage duration.

4. Discussion

The results in this study show that applying 1.8% H_2O_2 in the washing bath and dosing 300 L/h of 1.8% H_2O_2 in a 450 L washing

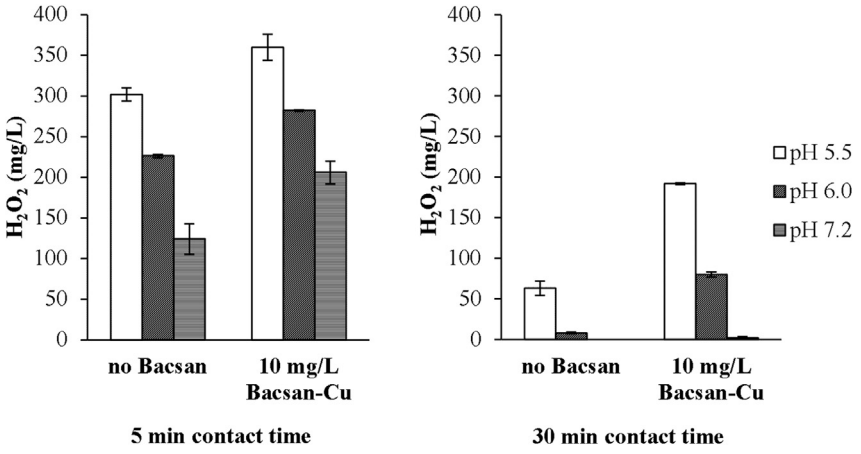


Fig. 7. Residual H_2O_2 concentration of initially added 590 mg/L H_2O_2, measured after addition of 1.5 mg/L catalase to phosphate-buffered solutions at different pH and in presence and absence of 10 mg/L Bacsan-Cu ($n = 3$).

bath is insufficient for maintaining the microbial wash water quality in the washing process when washing 333 ± 50 kg/h fresh-cut leafy vegetables. This, due to the build-up of organic matter in the washing bath which resulted in a rapid consumption of H_2O_2 due to oxidation of this organic matter. The exothermic nature of these oxidation reactions (Klais, 1993) explains the higher temperatures in the H_2O_2 treated washing bath compared to the untreated one.

Industrial washing of the leafy vegetables in ≤1.8% H_2O_2 for 1 min did not improve the decontamination efficiency compared to a water wash in this study. Other decontamination studies of fresh-cut leafy vegetables have been conducted with H_2O_2 in the range of 1–3%, with concentrations ≥2% showing improved decontamination efficiency compared to washing in water. However, these studies were performed at room or elevated temperature and with artificially inoculated microorganisms (Allwood et al., 2004; Huang & Chen, 2011; Lin et al., 2002). Possible explanations for higher removals in those experiments than in the present study are longer contact time, higher temperature, the use of artificial inocula, the use of specific bacterial pathogens instead of general plate counts, and the full-scale experiments in the present study *versus* lab-scale in the other studies. On the other hand, Ramos, Miller, Brandão, Teixeira, and Silva (2013) noted that at concentrations of 1–2%, H_2O_2 is not effective for produce decontamination. The issue of artificial inocula was illustrated by Hadjok et al. (2008), who used vacuum infiltration in order to achieve infiltration of inoculated *Salmonella* Montevideo in fresh-cut iceberg lettuce, and observed a much lower inactivation with H_2O_2/UV of internalized *Salmonella* than those bound to the surface. Improved decontamination efficiency of fresh-cut leafy vegetables with H_2O_2 in current industrial processes seems unrealistic, due to the requirement of long contact times, relatively high temperatures, high H_2O_2 wash water residual and the high reactivity of H_2O_2 with wash water organics. The observed increase in APC contamination on food contact surfaces illustrates that besides cross-contamination via the wash water, cross-contamination via food contact surfaces is an issue in fresh-cut leafy vegetables processing operations.

The executed case-study with UV/H_2O_2 in the fresh-cut leafy vegetables company shows the inadequacy of using an off-line disinfection technique to attempt to control the microbial contamination in a process with rapid and continuous influx of microbial contamination such as a fresh-cut leafy vegetables washing process. The issue is that an off-line disinfection technique only treats part of the water at any given time, whereas an *in situ* disinfection technique maintains a residual, as such treating all the water at any given time. From the point of food safety, pathogenic contamination is mostly not widely distributed among a batch of fresh-cut leafy vegetables, although there are extreme cases, for example 12.1% (4/33 positive samples) prevalence of *Salmonella* spp. on cabbage sampled in a field in India irrigated with partially treated municipal wastewater (Rai & Tripathi, 2007), or 76.9% (10/13) and 61.5% (8/13) prevalence of respectively *Cryptosporidium* spp. and *Giardia* spp. on lettuce sampled in a field in Spain irrigated with contaminated water from an irrigation canal. Nonetheless, the prevalence of bacterial pathogens (*Salmonella* spp., pathogenic *E. coli*, *Listeria monocytogenes*, *Campylobacter* spp.) is, as far as microbial screening studies show, much lower (<1% of the crops) (Johannessen, Loncarevic, & Kruse, 2002; University of Georgia, 2011). In those cases only a very minor part of the crops are contaminated. However, the statement that *in situ* disinfection is needed to avoid cross-contamination during point-contaminations holds for the same reasons as with a continuous microbial build-up, i.e. i) the off-line disinfection occurs at another location than the actual introduction of contamination in the wash water, and ii) the disinfection kinetics are too slow as again only part of the water will be treated at a certain time and the microbial contamination will rapidly disperse throughout the wash water.

The experiments in SWW showed that the stability of H_2O_2 was improved by the addition of Bacsan and to lesser extent Cu^{2+}. Elimination of heat labile molecules (80 °C, 10 min) greatly increased the stability of H_2O_2 in SWW, which was much less the case for free chlorine. The most obvious heat labile compound with a high and selective impact on H_2O_2 stability is catalase, originating from the lettuce tissue and present in the SWW. This study showed the inhibiting influence of Bacsan and to lesser extent Cu^{2+} on bovine liver catalase activity. The decrease in catalase activity in the presence of certain metals may be related to direct binding of metal ions (including Cu^{2+}, Zn^{2+}, Ag^{+}) to −SH groups of the catalase enzyme, as such inhibiting the enzyme (Atli, Alptekin, Tukel, & Canli, 2006; Atli & Canli, 2007). Furthermore, peroxidase (from vegetables) activity is reduced after heating (80 °C, 10 min) (Morales-Blancas, Chandia, & Cisneros-Zevallos, 2002), and inhibition of peroxidases by metal ions (Zn^{2+}, Ag^{+}) has been reported (Splittgerber & Tappel, 1979). As such it could be that peroxidases were inhibited by Bacsan. The less rapid H_2O_2 consumption by catalase at lower pH is attributed to catalase having to operate at sub-optimal pH. Bovine liver catalase has optimal activity at about pH 7 and isoelectric point (pI) of pH 5.4 (Maehly & Chance, 1954; Samejima, Kamata, & Shibata, 1962; Shi, Feng, Zhao, Guo, & Zhou, 2008), while lettuce catalase has optimal activity in the pH range

7–8, and consists of two izoenzymes (pI 5.8 and pI 6.2) (Bestwick, Adam, Puri, & Mansfield, 2001). Also, the H_2O_2 consumption rate is reduced at lower pH, especially below pH 3 (Ortiz, Angélica Rubio, & Lissi, 2000; Watts, Foget, Kong, & Teel, 1999) and the pH drops caused by the addition of Bacsan, and amplified when combined with H_2O_2, potentially slightly contributed to the inhibition of lettuce catalase in SWW.

Cu^{2+} in the presence of H_2O_2 generally does not induce free radical formation (through Fenton-like reactions) in systems that contain biomolecules because of the tendency of Cu^{2+} to tightly bind amino group containing compounds (Gutteridge & Wilkins, 1983; Pham, Xing, Miller, & Waite, 2013). This is an additional explanation why Cu^{2+} did not accelerate the H_2O_2 consumption. Therefore, the inactivation effect of H_2O_2/Cu^{2+} against bacteria is most likely due to the combined attack of the two disinfectants, rather than the production of radical formation (Macomber, Rensing, & Imlay, 2007; Orta De Velasquez et al., 2008).

In accordance with the increased stability of H_2O_2 when combined with Bacsan, synergistic effects were observed when these disinfectants were combined, both in SWW and in industrial leafy vegetables wash water. The lower inactivation of the APC in the industrial wash water, despite the higher H_2O_2 exposure, can be attributed to an overall more resistant microbiota than in the SWW, which is plausible, as APC is a non-discriminative enumeration method. The lower H_2O_2 consumption might be due to the fact that COD is a general parameter that measures the amount of oxygen that is necessary to oxidize the substances in the sample, and is used as an indicator for the organic load of the water. As such, it does not directly inform about the reaction rate of specific molecular species with H_2O_2 (as was observed to be significant in the heated SWW), nor the levels of iron or phosphate, which can also influence the H_2O_2 consumption (Watts et al., 1999). On the contrary, in a previous study, the COD was found to be a universal parameter that effectively predicted the disinfection efficiency of free chlorine to inactivate inoculated *E. coli* O157 in both SWW (made in the same fashion as in this study but with butterhead lettuce instead of iceberg lettuce) and fresh-cut leafy vegetables wash water from 2 processing companies (Van Haute, Sampers, Holvoet, et al., 2013). The chlorination trials in heated SWW in this study show the relative independence of chlorine consumption on specific heat labile compounds in the SWW.

The results in SWW confirm that H_2O_2 is a slow acting water disinfectant that quickly decomposes in the presence of high COD of fresh-cut lettuce origin. The inactivation of *E. coli* in oxidant demand free conditions requires a much higher exposure (concentration and contact time) to H_2O_2 (21) than to free chlorine (Rice, Clark, & Johnson, 1999; Van Haute, Sampers, Holvoet, et al., 2013; Zhao, Doyle, Zhao, Blake, & Wu, 2001) and ozone (Hunt & Mariñas, 1997). H_2O_2 is a non-radical reactive oxygen species that is capable of penetrating most biological membranes yet directly inactivate only few enzymes (Atli et al., 2006). It is the production of hydroxyl radicals through the Fenton-reaction with free intra- and extracellular iron that enables damage to membrane structures, DNA, and proteins (a. o. oxidation of Fe–S cluster proteins and more generally of cysteine residues in proteins) (Brudzynski, Abubaker, St-Martin, & Castle, 2011; Imlay, 2003; Raffellini et al., 2011).

Bacsan itself also showed antimicrobial activity when dosed at 10 mg/L (as Cu). Ionic copper and silver induce cell lysis and death by attaching to the negatively charged bacterial cell surface, disrupting cell wall permeability, blocking cell respiration, and causing extra- and intracellular protein denaturation (Agnihotri, Mukherji, & Mukherji, 2014; Feng et al., 2000; Huang et al., 2008; Lin, Vidic, Stout, McCartney, & Yu, 1998). Feng et al. (2000) observed that exposure of *E. coli* and *Staphylococcus aureus* cells to Ag^{+} led to transformation of the DNA into a condensed form, leading to loss of replication ability. Cu^{+}, Ag^{+}, and Zn^{2+} ions inactivate Fe–S cluster enzymes in *E. coli* and depletion of antioxidant reserves, particularly glutathione, can occur due to metal ions including Ag^{+} and Zn^{2+} and exposure of *E. coli* to toxic doses of these metal species can lead to depletion of total cellular thiols (Lemire, Harrison, & Turner, 2013; Macomber & Imlay, 2009). Also, combining sub-inhibitory concentrations of Ag^{+} with certain metal ions, including Cu^{2+} and Zn^{2+}, increased the toxicity of those metal ions on *E. coli* by a factor of 10 (Pedahzur et al., 1997). As such, the combination of measured ions in Bacsan exhibit antimicrobial action.

The synergistic microbial inactivation due to H_2O_2/Bacsan cannot be attributed to the presence of Cu^{2+} alone, but is dependent upon the combination of metal ions added to the SWW, in the presence of H_2O_2. Contrary to this study, synergy of H_2O_2 (50–250 mg/L) with 1 mg/L Cu^{2+} was observed for inactivation of vegetative bacteria in primary wastewater treatment effluent (Orta De Velasquez et al., 2008). Ag^{+}/H_2O_2 was more effective than H_2O_2 alone for inactivation of bacteria and fungi (Tote et al., 2009) and synergistically against *E. coli* (Batterman et al., 2000; Pedahzur et al., 2000, 1997). To the knowledge of the authors, this is the first study that considers the impact of water matrix catalase on H_2O_2 disinfection efficiency. Earlier research has shown that H_2O_2 is less effective against microorganisms with high catalase activity level (Armon, Laot, Lev, Shuval, & Fattal, 2000; Lambert, Johnston, & Simons, 1999; Sacchetti, De Luca, & Zanetti, 2009; Watts, Washington, Howsawkeng, Loge, & Teel, 2003). Monofunctional catalases are not only produced by plants and animals, but also widespread among bacteria (a. o. *E. coli*) and fungi, next to the catalase-peroxidases which are only distributed among bacteria and fungi, and the non-heme catalases that have only been found in certain bacterial species (Loewen, Klotz, & Hassett, 2000; Nadler, Goldberg, & Hochman, 1986). The synergy of H_2O_2 and Bacsan can be explained by i) the higher stability of H_2O_2 in SWW in the presence of Bacsan, allowing for higher exposure of the microorganisms to H_2O_2 and combined with the stress from Bacsan itself, ii) the multiple damage mechanism, meaning both disinfectants attack different targets in the microorganism, as such creating different stresses that could make it harder for the microorganism to remain viable compared to separate addition of the damage from both disinfectants (Koivunen & Heinonen-Tanski, 2005), and iii) the metal ions in Bacsan might inhibit the functioning of microbial catalases, as such rendering them more susceptible to H_2O_2 attack. Research on inhibition of microbial catalases of target microorganisms to improve the disinfection efficiency, as well as further research on inhibition of water matrix catalase might be interesting towards enhancing the use of H_2O_2 as a disinfectant.

It could be that in the case of washing in 10 mg/L Bacsan-Cu, Cu^{2+} was transferred to the lettuce in amounts that induced the activity of polyphenoloxidase, whereas a subsequent rinsing step leached the Cu^{2+} back from the lettuce and as such avoided additional browning (Vandekinderen, 2009). 500 mg/L of H_2O_2 does not influence the rate of fresh-cut lettuce browning and is much lower than the concentrations normally applied to decontaminate fruits and vegetables (mostly 1–5%), and the concentrations that potentially cause sensorial quality issues towards the lettuce (Lopez-Galvez, Ragaert, Palermo, Eriksson, & Devlieghere, 2013; McWatters, Chinnan, Walker, Doyle, & Lin, 2000; McWatters, Hashim, Walker, Doyle, & Rimal, 2002; Ukuku et al., 2012). In case H_2O_2/Bacsan would be implemented as a reconditioning technique for fresh-cut leafy vegetables wash water, most of the Cu^{2+} (from the Bacsan) would remain in the SWW after reconditioning, and could come into contact with the fresh-cut leafy vegetables when the water is reused. This is advantageous from a cost perspective, because it would enable the reuse of the metal

ions for water disinfection. Nonetheless, implementation of a rinsing step with tap water would be required to avoid presence of Cu^{2+} on the packaged lettuce. Furthermore, regulations (for Cu) or guidelines (for Ag and Cu) exist to govern their presence in drinking water (Council of the European Union, 1998; USEPA, 2009, 2013; WHO, 2003, 2004) to avoid ingestion of aesthetically altering (discoloration of the skin and white part of eye in the case of Ag intake) or toxic dosages of these metals. From the perspective of the fresh-cut produce processor and the consumer safety, the primary concern is the possible transfer of these metals to the fresh-cut leafy vegetables and the influence of a rinsing step on the removal of these metals from the fresh-cut leafy vegetables; more so than directly assessing the quality of the wash water itself as this is not consumed.

5. Conclusions

H_2O_2 is not suited as an *in situ* wash water disinfectant to avoid cross-contamination due to the slow water disinfection kinetics and the rapid consumption in fresh-cut leafy vegetables water. As such, excessive amounts of H_2O_2 would have to be dosed to maintain the microbial wash water quality. The use of off-line disinfection systems (such as the UV/H_2O_2 system applied in this study) are incapable of avoiding cross-contamination in a fresh-cut leafy vegetables washing process, due to the fact that at any given time only part of the wash water is being 'controlled', i.e. disinfected. When combined with Bacsan, H_2O_2 showed increased stability in SWW and industrial wash water and synergistic inactivation of *E. coli* and APC in SWW and of APC in industrial wash water. As such, H_2O_2/Bacsan shows potential for use in reconditioning processes, when the inactivation rate is of less importance. The metal ions mixture (Cu^{2+}, Zn^{2+}, Ag^{+}) in Bacsan stabilizes H_2O_2 through a mechanism that most likely involves the inhibition of lettuce catalase present in the fresh-cut leafy vegetables wash water, and potentially other heat labile compounds. When combined with Ag^{+}, the H_2O_2 stabilizing action of Bacsan is further enhanced in a synergistic fashion. Further research towards the combination of H_2O_2 and metal ion mixtures such as Bacsan could greatly improve the potential of H_2O_2 as a water disinfectant in general.

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References

Agnihotri, S., Mukherji, S., & Mukherji, S. (2014). Size-controlled silver nanoparticles synthesized over the range 5–100 nm using the same protocol and their antibacterial efficacy. *RSC Advances, 4*, 3974–3983.

Allwood, P. B., Malik, Y. S., Hedberg, C. W., & Goyal, S. M. (2004). Effect of temperature and sanitizers on the survival of feline calicivirus, *Escherichia coli*, and F-specific coliphage MS2 on leafy salad vegetables. *Journal of Food Protection, 67*(7), 1451–1456.

Anderson, J. A. (2002). Catalase activity, hydrogen peroxide content and thermotolerance of pepper leaves. *Scientia Horticulturae, 95*(4), 277–284.

Armon, R., Laot, N., Lev, O., Shuval, H., & Fattal, B. (2000). Controlling biofilm formation by hydrogen peroxide and silver combined disinfectant. *Water Science and Technology, 42*(1–2), 187–192.

Artés, F., Gómez, P., Aguayo, E., Escalona, V., & Artés-Hernández, F. (2009). Sustainable sanitation techniques for keeping quality and safety of fresh-cut plant commodities. *Postharvest Biology and Technology, 51*(3), 287–296.

Atli, G., Alptekin, O., Tukel, S., & Canli, M. (2006). Response of catalase activity to Ag^{+}, Cd^{2+}, Cr^{6+}, Cu^{2+} and Zn^{2+} in five tissues of freshwater fish *Oreochromis niloticus*. *Comparative Biochemistry and Physiology: Part C Toxicology & Pharmacology, 143*(2), 218–224.

Atli, G., & Canli, M. (2007). Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 145*(2), 282–287.

Barbee, S. L., Weber, D. J., Sobsey, M. D., & Rutala, W. A. (1999). Inactivation of *Cryptosporidium parvum* oocyst infectivity by disinfection and sterilization processes. *Gastrointestinal Endoscopy, 49*(5), 605–611.

Batterman, S., Zhang, L., & Wang, S. (2000). Quenching of chlorination disinfection by-product formation in drinking water by hydrogen peroxide. *Water Research, 34*(5), 1652–1658.

Bestwick, C. S., Adam, A. L., Puri, N., & Mansfield, J. W. (2001). Characterisation of and changes to pro and anti-oxidant enzyme activities during the hypersensitive reaction in lettuce (*Lactuca sativa* L.). *Plant Science, 161*(3), 497–506.

Brudzynski, K., Abubaker, K., St-Martin, L., & Castle, A. (2011). Re-examining the role of hydrogen peroxide in bacteriostatic and bactericidal activities of honey. *Front Microbiology, 2*, 213.

Council of the European Union. (1998). *Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption*. Brussels, Belgium: European Council. Available http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri_CELEX:31998L0083:EN:NOT. Last accession: 24.08.13.

Feng, Q. L., Wu, J., Chen, G. Q., Cui, F. Z., Kim, T. N., & Kim, J. O. (2000). A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. *Journal of Biomedical Materials Research, 52*(4), 662–668.

Gutteridge, J. M. C., & Wilkins, S. (1983). Copper salt-dependent hydroxyl radical formation – damage to proteins acting as anti-oxidant. *Biochimica et Biophysica Acta, 759*(1–2), 38–41.

Hadjok, C., Mittal, G. S., & Warriner, K. (2008). Inactivation of human pathogens and spoilage bacteria on the surface and internalized within fresh produce by using a combination of ultraviolet light and hydrogen peroxide. *Journal of Applied Microbiology, 104*(4), 1014–1024.

Hirvi, Y., Griffiths, M. W., McKellar, R. C., & Modler, H. W. (1996). Linear-transform and non-linear modelling of bovine milk catalase inactivation in a high-temperature short-time pasteurizer. *Food Research International, 29*(1), 89–93.

Holvoet, K., Jacxsens, L., Sampers, I., & Uyttendaele, M. (2012). Insight into the prevalence and distribution of microbial contamination to evaluate water management in the fresh produce processing industry. *Journal of Food Protection, 75*(4), 671–681.

Holvoet, K., Sampers, I., Callens, B., Dewulf, J., & Uyttendaele, M. (2013). Moderate prevalence of antimicrobial resistance in *Escherichia coli* isolates from lettuce, irrigation water, and soil. *Applied and Environmental Microbiology, 79*(21), 6677–6683.

Huang, Y., & Chen, H. (2011). Effect of organic acids, hydrogen peroxide and mild heat on inactivation of *Escherichia coli* O157:H7 on baby spinach. *Food Control, 22*(8), 1178–1183.

Huang, H. I., Shih, H. Y., Lee, C. M., Yang, T. C., Lay, J. J., & Lin, Y. E. (2008). In vitro efficacy of copper and silver ions in eradicating *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii*: implications for on-site disinfection for hospital infection control. *Water Research, 42*(1–2), 73–80.

Hunt, N. K., & Mariñas, B. J. (1997). Kinetics of *Escherichia coli* inactivation with ozone. *Water Research, 31*(6), 1355–1362.

Imlay, J. A. (2003). Pathways of oxidative damage. *Annual Review of Microbiology, 57*, 395–418.

ISO. (1999). *Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium* (ISO 6222:1999).

ISO. (2000a). *Water quality – Detection and enumeration of Escherichia coli and coliform bacteria – Part 1: Membrane filtration method* (ISO 9308–1:2000).

ISO. (2000b). *Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method* (ISO 7899–2).

ISO. (2003). *Microbiology of food and animal feeding stuffs-horizontal method for the enumeration of microorganisms-colony count technique at 30°C* (ISO 4833:2003).

ISO. (2006). *Water quality – Sampling for microbiological analysis* (ISO 19458:2006).

Johannessen, G. S., Loncarevic, S., & Kruse, H. (2002). Bacteriological analysis of fresh produce in Norway. *International Journal of Food Microbiology, 77*(3), 199–204.

Keskinen, L. A., Burke, A., & Annous, B. A. (2009). Efficacy of chlorine, acidic electrolyzed water and aqueous chlorine dioxide solutions to decontaminate *Escherichia coli* O157:H7 from lettuce leaves. *International Journal of Food Microbiology, 132*(2–3), 134–140.

Klais, O. (1993). Hydrogen peroxide decomposition in the presence of organic material: a case study. *Thermochimica Acta, 225*(2), 213–222.

Klassen, N. V., Marchington, D., & McGowan, H. C. E. (1994). H_2O_2 determination by the I_3^- method and by $KMnO_4$ titration. *Analytical Chemistry, 66*(18), 2921–2925.

Koivunen, J., & Heinonen-Tanski, H. (2005). Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments. *Water Research, 39*(8), 1519–1526.

Lambert, R. J., Johnston, M. D., & Simons, E. A. (1999). A kinetic study of the effect of hydrogen peroxide and peracetic acid against *Staphylococcus aureus* and *Pseudomonas aeruginosa* using the bioscreen disinfection method. *Journal of Applied Microbiology, 87*(5), 782–786.

Lemire, J. A., Harrison, J. J., & Turner, R. J. (2013). Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nature Reviews Microbiology, 11*(6), 371–384.

Li, D., Baert, L., De Jonghe, M., Van Coillie, E., Ryckeboer, J., Devlieghere, F., et al. (2011). Inactivation of murine norovirus 1, coliphage phiX174, and *Bacteroides fragilis* phage B40-8 on surfaces and fresh-cut iceberg lettuce by hydrogen peroxide and UV light. *Applied and Environmental Microbiology, 77*(4), 1399–1404.

Lin, C.-M., Moon, S. S., Doyle, M. P., & McWatters, K. H. (2002). Inactivation of *Escherichia coli* O157:H7, *Salmonella enterica* serotype Enteritidis, and *Listeria monocytogenes* on lettuce by hydrogen peroxide and lactic acid and by hydrogen peroxide with mild heat. *Journal of Food Protection, 65*(8), 1215–1220.

Lin, Y.-s. E., Vidic, R. D., Stout, J. E., McCartney, C. A., & Yu, V. L. (1998). Inactivation of *Mycobacterium avium* by copper and silver ions. *Water Research, 32*(7), 1997–2000.

Loewen, P. C., Klotz, M. G., & Hassett, D. J. (2000). Catalase—an "old" enzyme that continues to surprise us. *ASM News, 66*(2), 76–82.

López-Gálvez, F., Gil, M. I., Truchado, P., Selma, M. V., & Allende, A. (2010). Cross-contamination of fresh-cut lettuce after a short-term exposure during pre-washing cannot be controlled after subsequent washing with chlorine dioxide or sodium hypochlorite. *Food Microbiology, 27*(2), 199–204.

Lopez-Galvez, F., Ragaert, P., Palermo, L. A., Eriksson, M., & Devlieghere, F. (2013). Effect of new sanitizing formulations on quality of fresh-cut iceberg lettuce. *Postharvest Biology and Technology, 85*(0), 102–108.

Macomber, L., & Imlay, J. A. (2009). The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proceedings of the National Academy of Sciences of the United States of America, 106*(20), 8344–8349.

Macomber, L., Rensing, C., & Imlay, J. A. (2007). Intracellular copper does not catalyze the formation of oxidative DNA damage in *Escherichia coli*. *Journal of Bacteriology, 189*(5), 1616–1626.

Maehly, A. C., & Chance, B. (1954). The assay of catalases and peroxidases. *Methods of Biochemical Analysis, 1*, 357–424.

McWatters, L. H., Chinnan, M. S., Walker, S. L., Doyle, M. P., & Lin, C. M. (2002). Consumer acceptance of fresh-cut iceberg lettuce treated with 2% hydrogen peroxide and mild heat. *Journal of Food Protection, 65*(8), 1221–1226.

McWatters, K. H., Hashim, I. B., Walker, S. L., Doyle, M. P., & Rimal, A. P. (2002). Acceptability of lettuce treated with a lactic acid and hydrogen peroxide antibacterial solution. *Journal of Food Quality, 25*(3), 223–242.

Morales-Blancas, E. F., Chandia, V. E., & Cisneros-Zevallos, L. (2002). Thermal inactivation kinetics of peroxidase and lipoxygenase from broccoli, green asparagus and carrots. *Journal of Food Science, 67*(1), 146–154.

Nadler, V., Goldberg, I., & Hochman, A. (1986). Comparative study of bacterial catalases. *Biochimica et Biophysica Acta (BBA) – General Subjects, 882*(2), 234–241.

Olaimat, A. N., & Holley, R. A. (2012). Factors influencing the microbial safety of fresh produce: a review. *Food Microbiology, 32*(1), 1–19.

Orta De Velasquez, M. T., Yanez-Noguez, I., Jimenez-Cisneros, B., & Luna Pabello, V. M. (2008). Adding silver and copper to hydrogen peroxide and peracetic acid in the disinfection of an advanced primary treatment effluent. *Environmental Technology, 29*(11), 1209–1217.

Ortiz, V., Angélica Rubio, M., & Lissi, E. A. (2000). Hydrogen peroxide deposition and decomposition in rain and dew waters. *Atmospheric Environment, 34*(7), 1139–1146.

Parish, M. E., Beuchat, L. R., Suslow, T. V., Harris, L. J., Garrett, E. H., Farber, J. N., et al. (2003). Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety, 2*, 161–173.

Pedahzur, R., Katzenelson, D., Barnea, N., Lev, O., Shuval, H. I., Fattal, B., et al. (2000). The efficacy of long-lasting residual drinking water disinfectants based on hydrogen peroxide and silver. *Water Science and Technology, 42*(1–2), 293–298.

Pedahzur, R., Shuval, H. I., & Ulitzur, S. (1997). Silver and hydrogen peroxide as potential drinking water disinfectants: their bactericidal effects and possible modes of action. *Water Science and Technology, 35*(11–12), 87–93.

Pham, A. N., Xing, G. W., Miller, C. J., & Waite, T. D. (2013). Fenton-like copper redox chemistry revisited: hydrogen peroxide and superoxide mediation of copper-catalyzed oxidant production. *Journal of Catalysis, 301*, 54–64.

Raffellini, S., Guerrero, S., & Alzamora, S. M. (2008). Effect of hydrogen peroxide concentration and pH on inactivation kinetics of *Escherichia coli*. *Journal of Food Safety, 28*(4), 514–533.

Raffellini, S., Schenk, M., Guerrero, S., & Alzamora, S. M. (2011). Kinetics of *Escherichia coli* inactivation employing hydrogen peroxide at varying temperatures, pH and concentrations. *Food Control, 22*(6), 920–932.

Rai, P. K., & Tripathi, B. D. (2007). Microbial contamination in vegetables due to irrigation with partially treated municipal wastewater in a tropical city. *International Journal of Environmental Health Research, 17*(5), 389–395.

Ramos, B., Miller, F. A., Brandão, T. R. S., Teixeira, P., & Silva, C. L. M. (2013). Fresh fruits and vegetables—An overview on applied methodologies to improve its quality and safety. *Innovative Food Science & Emerging Technologies, 20*(0), 1–15.

Rice. (1999).

Sacchetti, R., De Luca, G., & Zanetti, F. (2009). Control of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* contamination of microfiltered water dispensers with peracetic acid and hydrogen peroxide. *International Journal of Food Microbiology, 132*(2–3), 162–166.

Samejima, T., Kamata, M., & Shibata, K. (1962). Dissociation of bovine liver catalase at low pH. *Journal of Biochemistry, 51*, 181–187.

Sapers, G. M. (2001). Efficacy of washing and sanitizing methods for disinfection of fresh fruit and vegetable products. *Food Technology and Biotechnology, 39*(4), 305–312.

Shi, X., Feng, M., Zhao, Y., Guo, X., & Zhou, P. (2008). Overexpression, purification and characterization of a recombinant secretary catalase from *Bacillus subtilis*. *Biotechnology Letters, 30*(1), 181–186.

Splittgerber, A. G., & Tappel, A. L. (1979). Inhibition of glutathione-peroxidase by cadmium and other metal-ions. *Archives of Biochemistry and Biophysics, 197*(2), 534–542.

Tofant, A., Vučemilo, M., Pavičić, Ž., & Milić, D. (2006). The hydrogen peroxide, as a potentially useful slurry disinfectant. *Livestock Science, 102*(3), 243–247.

Toledo, R. T., Escher, F. E., & Ayres, J. C. (1973). Sporicidal properties of hydrogen peroxide against food spoilage organisms. *Applied Microbiology, 26*(4), 592–597.

Tomas-Callejas, A., Lopez-Galvez, F., Sbodio, A., Artes, F., Artes-Hernandez, F., & Suslow, T. V. (2012). Chlorine dioxide and chlorine effectiveness to prevent *Escherichia coli* O157:H7 and *Salmonella* cross-contamination on fresh-cut Red Chard. *Food Control, 23*(2), 325–332.

Tote, K., Vanden Berghe, D., Levecque, S., Benere, E., Maes, L., & Cos, P. (2009). Evaluation of hydrogen peroxide-based disinfectants in a new resazurin microplate method for rapid efficacy testing of biocides. *Journal of Applied Microbiology, 107*(2), 606–615.

Ukuku, D. O., Bari, L., & Kawamoto, S. (2012). Hydrogen peroxide. In *Decontamination of fresh and minimally processed produce* (pp. 197–214). Wiley-Blackwell.

United States Environmental Protection Agency. (1997). *Evaluation of the efficacy of a new secondary disinfectant formulation using hydrogen peroxide and silver and the formulation of disinfection products resulting from interactions with conventional disinfectants*. Available http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/198/report/F. Last accession: 26.08.13.

United States Environmental Protection Agency. (2013). *National secondary drinking water regulations*. Available http://water.epa.gov/drink/contaminants/secondarystandards.cfm. Last accession: 03.07.13.

United States Environmental Protection Agency. (May 2009). *National primary drinking water regulations*. EPA 816-F-09–0004. Available http://water.epa.gov/drink/contaminants/index.cfm. Last accession: 03.07.13.

University of Georgia. (2011). *Epidemiological, prevalence, and economic data associated with leafy green produce contamination*. Center for Food Safety. Available http://www.ugacfs.org/producesafety/Pages/Basics/SurveyLeafyGreens.html. Last accession: 12.07.13.

Van Haute, S., Sampers, I., Holvoet, K., & Uyttendaele, M. (2013). Physicochemical quality and chemical safety of chlorine as a reconditioning agent and wash water disinfectant for fresh-cut lettuce washing. *Applied and Environmental Microbiology, 79*(9), 2850–2861.

Van Haute, S., Sampers, I., Jacxsens, L., & Uyttendaele, M. (2013). Selection criteria for water disinfection techniques in agricultural practices. *Critical Reviews in Food Science and Nutrition*. http://dx.doi.org/10.1080/10408398.2012.705360 (Epub ahead of print Nov 26, 2013).

Vandekinderen, I. (2009). *Decontamination treatments for fresh-cut vegetables and their effects on microbial and sensory quality, physiology and nutrient content*. Doctoral dissertation. Ghent: Faculty of Bioscience Engineering, Ghent University.

Watts, R. J., Foget, M. K., Kong, S.-H., & Teel, A. L. (1999). Hydrogen peroxide decomposition in model subsurface systems. *Journal of Hazardous Materials, 69*(2), 229–243.

Watts, R. J., Washington, D., Howsawkeng, J., Loge, F. J., & Teel, A. L. (2003). Comparative toxicity of hydrogen peroxide, hydroxyl radicals, and superoxide anion to *Escherichia coli*. *Advances in Environmental Research, 7*(4), 961–968.

Weir, S. C., Pokorny, N. J., Carreno, R. A., Trevors, J. T., & Lee, H. (2002). Efficacy of common laboratory disinfectants on the infectivity of *Cryptosporidium parvum* oocysts in cell culture. *Applied Environmental Microbiology, 68*(5), 2576–2579.

World Health Organization. (2003). *Silver in drinking-water. Background document for preparation of WHO guidelines for drinking-water quality* (WHO/SDE/WSH/03.04/14). Geneva: World Health Organization. Available http://www.who.int/water_sanitation_health/dwq/chemicals/silver.pdf. Last accession: 04.07.13.

World Health Organization. (2004). *Copper in drinking-water. Background document for preparation of WHO guidelines for drinking-water quality* (WHO/SDE/WSH/03.04/88). Geneva: World Health Organization. Available http://www.who.int/water_sanitation_health/dwq/chemicals/coppersum.pdf. Last accession: 04.07.13.

Zhao, T., Doyle, M. P., Zhao, P., Blake, P., & Wu, F. M. (2001). Chlorine inactivation of *Escherichia coli* O157:H7 in water. *Journal of Food Protection, 64*(10), 1607–1609.